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Research report

Abundant expression of zinc transporters in the amyloid plaques of Alzheimer's disease brain

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ABSTRACT

The pathological key features of Alzheimer's disease (AD) are β-amyloid peptide (Aβ)-containing senile plaques (SP) and neurofibrillary tangles. Previous studies have suggested that an extracellular elevation of the zinc concentration can initiate the deposition of Aβ and lead to the formation of SP. In the present study, we present data showing a correlation between zinc ions, zinc transporters (ZNTs) and AD, using immersion autometallography (AMG) and double immunofluorescence for the ZNTs and Aβ. We found that all the ZNTs tested (ZNT1, 3, 4, 5, 6, 7) were extensively present in the Aβ-positive plaques in the cortex of human AD brains, and the density of autometallographic silver enhanced zinc–sulphur nanoparticles were much higher in the plaques than in the surrounding zinc enriched (ZEN) terminals. Moreover, we found an abundant expression of ZNT3 and autometallographic grains in the amyloid angiopathic vessels. The subcellular localization of ZNTs and zinc ions were not detected, due to the limited tissue preservation in the present study. In conclusion, our data provided significant morphological evidence of zinc ions and ZNTs being actively involved in the pathological processes that lead to plaque formation.

Alzheimer's disease (AD) is the most common dementia in Western societies. It is clinically characterized by a progressive loss of cognitive function. The hallmark pathological findings include widespread neuron loss, formation of extracellular β-amyloid peptide (Aβ)-containing senile plaques (SP) and intracellular neurofibrillary tangles. Up to now the cause of progression and pathology of AD is far from fully elucidated. An impressive amount of AD research has focused upon the toxicological effects of the Aβ peptide deposited in the AD brain [5,28,29,45,51,53,77]. Among the numerous pathogenic mechanisms suggested to cause SP, the role of zinc in the initiation and formation of SP have recently gained considerable attention [66,69].

In the nervous system, most zinc ions are firmly bound to macromolecules. Only a fraction of the total amount of zinc ions in the brain is present as free or loosely bound. Only these chelatable zinc ions can be histochemically detected. Two methods are widely used for detecting chelatable zinc ions in tissues. One is using fluorescent chelator to bind zinc ions and trace the zinc bound fluorescent dye at low light microscopic magnifications [25,79]. The other is to transform zinc ions into zinc–sulphur nanocrystals (immersion, *in vitro*) or zinc–selenium nanocrystals (*in vivo*). These nanocrystal signals can be silver enhanced by autometallography (AMG) on tissue sections and analyzed at both light and electron microscopic levels [20].

Body zinc homeostasis results from a coordinated regulation of intestinal uptake, fecal elimination of excess zinc, renal reabsorption, and bone and other organ storage [17,37]. Zinc ions cannot cross biological membranes by passive diffusion. Zinc trafficking is mediated by zinc transporters (ZNTs and ZIPs) [15,41]. ZNTs (SLC30) function to either transport zinc ions into intracellular compartments for zinc sequestration or export of zinc out of cells in a zinc excess condition. Eight members of the ZNT family have been cloned and are referred as to ZNT1-8 [13,31,32,33,36,54,55,60]. Two other ZNT genes, ZNT9 and ZNT10, have been predicted from the mouse and human genome resources [60,62]. All ZNT proteins have similar topology, with six transmembrane domains, an intracellular N-terminus and C-terminus and a His-rich loop

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between transmembrane domains IV and V [27,30,57], except for ZNT5 which has extra 10 predicted transmembrane domains at the N-termini of the protein and the His-rich loop locates between transmembrane domains XIV and XV. The His-rich loop region has been predicted to be the zinc-binding site [4,50].

Several lines of studies show that zinc is involved in AD neuropathology as the changes in the distribution and level of the labile zinc pool in neurons are observed in AD brains [19,23,58,64,67]. The regions of the brain queued to have Aβ-plaque deposition (neocortex, hippocampal region and amygdala) are densely populated with zinc enriched (ZEN) terminals with loads of zinc-containing synaptic vesicles, whereas regions with few Aβ-plaques (cerebellum, brainstem and thalamus) have fewer ZEN terminals.

Aβ possesses selective high and low affinity zinc binding sites [24]. An elevation of zinc ion level can ignite deposition of Aβ and leads to SP [8,9]. Metal chelating agents (Clioquinol and DP-109, both membrane permeable chelators) have been suggested inhibiting the formation of amyloid plaques in APP transgenic mouse brain [7,11,40]. Therefore, it has been hypothesized that a disturbance of brain zinc ion homeostasis plays an important role in the AD brain.

Although much has been learned about the involvement of zinc ions in the pathological process of AD, little is known about locations of ZNTs in the human brain, and that is in particular true for brains suffering from AD. Recent evidences suggest that significant alterations in the expression of ZNT1 [44], ZNT4 and ZNT6 [43,44,63] in human AD brains. In order to further evaluate the roles of ZNTs in the AD pathogenesis, we studied the distributions of ZNT1, ZNT3, ZNT4, ZNT5, ZNT6, ZNT7 and zinc ions in the human brain affected by AD by means of immunofluorescence and immersion AMG.

1. Experimental procedures

1.1. Antibodies

All ZNT antibodies used in this study were affinity-purified rabbit anti-sera specific for each ZNT protein. ZNT1 anti-serum was kindly provided by Dr. W.F. Silverman [59]; ZNT3 anti-serum was kindly provided by Dr. R.D. Palmiter [55]. The mouse monoclonal antibody detecting amino acid residues 1–12 of human Aβ was purchased from Sigma. Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG, Texas Red-conjugated donkey anti-mouse IgG, and normal donkey serum (NDS) were purchased from Jackson ImmunoResearch Laboratory (Pennsylvania, USA). Specificities of the primary antibodies against ZNT1 and ZNT3-7 were described previously by Western blot analyses [32,36,55,59,78].

1.2. Brain specimens and tissue preparation

Postmortem tissues of cerebral cortex from 5 patients (3 males, 2 females average age 80 years) with a definitive diagnosis of AD were obtained from the Alzheimer's Disease Research Center at Johns Hopkins University, Baltimore, Maryland, USA. The brain samples were placed either in a 4% paraformaldehyde solution for immunofluorescence analysis or in a 3% glutaraldehyde solution for AMG analysis.

1.3. Immunofluorescence

Brain tissues were dehydrated in graded alcohols and xylene and embedded in paraffin. Paraffin sections, 10-μm-thick, were cut and mounted on glass slides. Prior to immunofluorescence staining, the sections were dewaxed in xylene and rehydrated through graded alcohols. Sections were then rinsed in a 0.1 M Tris–HCl buffered saline (TBS, pH 7.4) and boiled in a TEG buffer (50 mM Tris–HCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethanesulphonyl fluoride) for 5 min in a microwave. After cooling and rinsing in TBS, sections were pre-incubated with NDS (1:20) for 1 h and then incubated overnight in a mixture of primary antibodies, mouse anti-Aβ (1:500) and rabbit anti-ZNT1 (1:50), ZNT3 (1:50), ZNT4 (1:25), ZNT5 (1:25), ZNT6 (1:50) or ZNT7 (1:25). After rinsing, sections were incubated for 2 h with a mixture of secondary antibodies, FITC-conjugated donkey anti-rabbit IgG (1:50, for labeling one of the ZNTs) and Texas Red-conjugated donkey anti-mouse IgG (1:50, for labeling Aβ). After rinsing with PBS, sections were mounted with an anti-fading mounting medium and examined using a confocal laser scanning microscope (SP2, Leica). Images were collected and processed using an Adobe Photoshop program.

To assess nonspecific staining, a few sections in every experiment were incubated with NDS instead of primary antibodies followed by all subsequent procedures as described above. No distinct staining was observed (data not shown).

1.4. Immersion AMG

Brain tissue slices, 1- and 2-mm-thick, were prepared with a "fast tissue slicer" (Histotech, Denmark) and immersed in NeoTimm solution (0.1% sodium sulphide and 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). Brain slices were kept at 4 °C for 3 days. After rinsing with 0.1 M phosphate buffer, these slices were placed in a 30% sucrose solution at 4 °C until they sank to the bottom of the jar. Brain slices were then frozen with CO_2 and cut in 30-μm sections with a cryostat. Sections were incubated in an AMG developer for 60 min at 26 °C, counterstained with toluidine blue, dehydrated in alcohol and xylene, and covered with a cover slide [16,20,21,64]. These brain slices were then analyzed and photographed with a light microscope. In order to ensure the specificity of the zinc staining, the sodium diethyldithiocarbamate trihydrate (Merck) control procedure was performed as described previously [18,20,64].

2. Results

2.1. Double immunofluorescence labeling of Aβ and ZNTs

Double immunofluorescence for Aβ and ZNT1, ZNT3, ZNT4, ZNT5, ZNT6 or ZNT7 was performed to analyze the distribution and correlation of Aβ and ZNTs in the AD brains. Low magnification (Fig. 1a1–f1) demonstrated numerous plaques of different size and density stained with Aβ and ZNTs antibodies throughout the cerebral cortex in the AD brain. At higher magnification, the majority of the plaques labeled by Aβ demonstrated typical characteristics of a compact plaque that contained densely packed Aβ fibrils throughout the plaque (Fig. 1a4–f4). ZNT1 and ZNT3-7 immunostaining were abundantly present in the Aβ-positive plaque, but the intensity and localization of the various transporters differed within the individual plaque as well as between plaques (Fig. 1a2 and 3, f2 and 3). Among the ZNTs examined, ZNT5 showed the strongest immunoreaction in the Aβ-positive plaques. Furthermore, ZNT3 immunofluorescence could be seen in the amyloid angiopathic vessels (Fig. 1b2–b4). The subcellular localization of ZNTs could not be present precisely, due to the limited tissue preservation.

2.2. Distribution of zinc ions in the senile plaques

AMG staining of the zinc–sulphur nanocrystals created by immersion of the sections in a glutaraldehyde solution saturated with sodium sulphide [20] was used to demonstrate the distribution of free zinc ions in the cortex of AD brains. In the light microscope analysis, zinc ions were more abundant in the plaques than in the surrounding ZEN terminals that were enriched in the neocortex. The diameters of the plaques were between 10 and 100 μM (Fig. 2a). At a higher magnification, the centers of most AMG stained plaques were black and irregular. It appeared to have processes emanating from the dense center and radiating to the periphery of the plaques as previously described in mice [64,65]. The rest of the plaques materially resembled the appearance of a dusty cloud (Fig. 2b and d). Occasionally, the whole plaque was composed of a wickerwork of fine AMG stained fibers and totally void of the dark-stained center (Fig. 2e). Moreover, large amounts of AMG enhanced zinc–sulphur nanocrystals were seen in some blood vessel walls (Fig. 2c and f) of the AD brains.

3. Discussion

The pathological key feature of AD is the development of Aβ-containing SP. An increase of zinc ions in the brain is believed to induce the deposition of Aβ by directly binding to Aβ through histidine bridges leading to denaturalization of the protein and

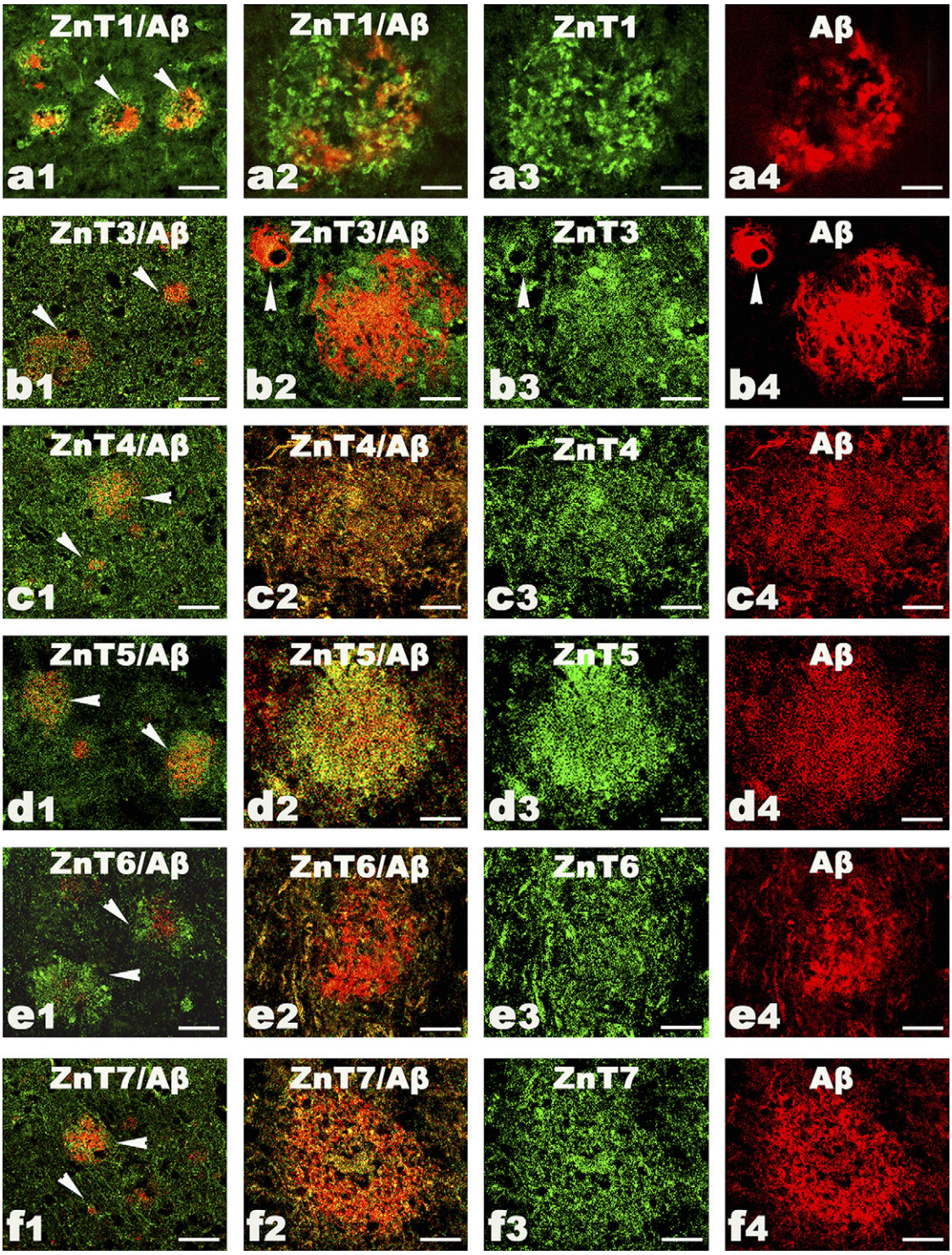

Fig. 1. Double immunofluorescence analysis of Aβ and ZNT1 (a), ZNT3 (b), ZNT4 (c), ZNT5 (d), ZNT6 (e), or ZNT7 (f) in the cerebral cortex of human AD brains. Low magnification shows abundant expression of ZNTs in the numerous Aβ-positive plaques (arrowheads in a1, b1, c1, d1, e1, f1). High magnification shows an extensive distribution of ZNTs (a3–f3) in the SP. ZNT3 immunoreactivity is also expressed within the walls and in the vicinity of cerebral vessels (arrowhead in b2–b4). The majority of the plaques labeled by Aβ demonstrate the typical characteristics of a compact plaque that contains densely packed Aβ fibrils throughout the plaques (a4–f4). Scale bars = 150 μm (a1–f1), 15 μm (a2–4 to f2–4).

formation of senile plaques [8,9]. In the present study, immunofluoresence staining of human AD brain sections revealed an abundant expression of ZNTs in numerous Aβ-positive plaques throughout the cerebral cortex. Moreover, we showed that abundant zinc ions were detected in the senile plaques, supporting the notion that zinc is an important player in the creation of and, possibly, maintenance of senile plaques.

Six ZNT family members examined were expressed in the Aβ-positive zinc enriched plaques. Previous studies from our and other laboratories have demonstrated that different zinc trans-

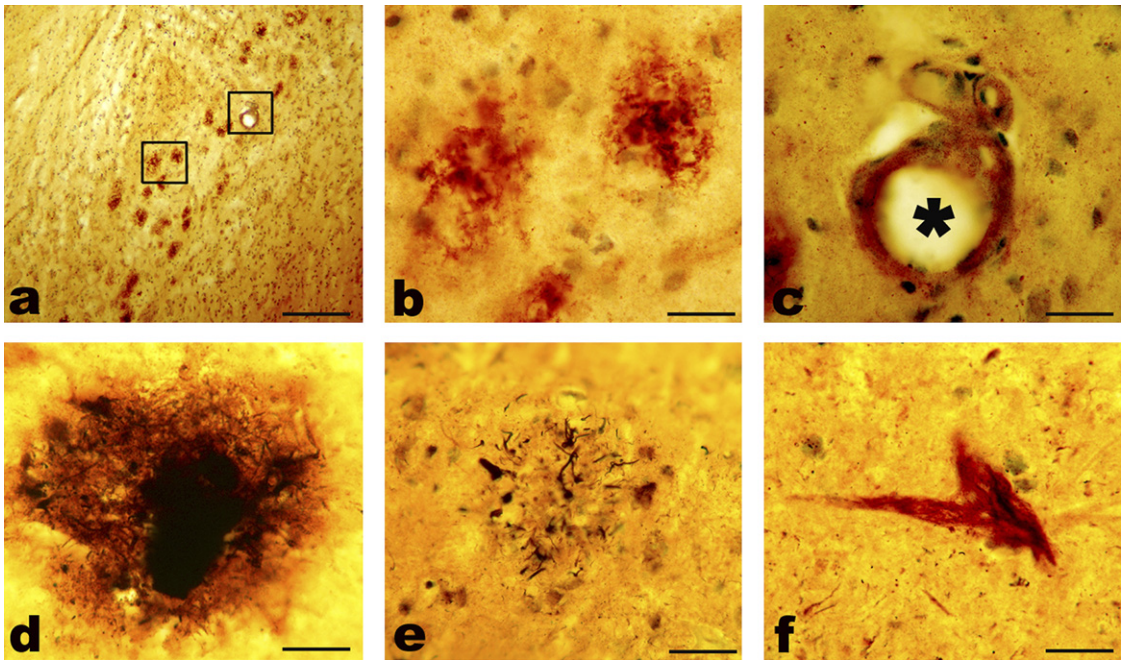

Fig. 2. AMG staining of zinc ions within the cortex of human AD brains. Zinc is more abundant in the plaques and the blood vessel walls than in the surrounding area. The AMG-positive plaques are distributed throughout the cortex (a). High magnification shows the AMG-positive plaques (b, d, e) and blood vessels (c and f). Most plaques are shown with dense cores and processes emanating from dense cores (b and d). Few plaques are composed of a feltwork of fine fibers and are absent from the dark-stained center (e). A large amount of zinc ions can also be seen in the walls of blood vessels (f). Scale bars = 200 μm (a), 20 μm (b–f). Asterisks (*) indicate the lumen of blood vessels.

porter proteins have different cellular and subcellular localizations. The difference in cellular localization may correlate to their roles in cellular zinc homeostasis both under normal and pathological conditions [15,33,71,72,73,74]. It also suggests a much complicated mechanism by which ZNTs are involved in the plaque formation.

ZNT1, a ubiquitous zinc transporter localized in the plasma membrane, functions to efflux zinc out of cells [1,52,56,59]. Exposure to a high dose of zinc or oral administration of zinc up-regulates *Znt1* gene expression in cultured cells [38,70] or in tissues isolated from mice such as small intestine, kidney, and liver [22,41,47]. Therefore, we hypothesize that a disruption in cellular zinc homeostasis in neuronal cells triggers up-regulation of *Znt1* transcription and protein expression to prevent neurons from death. Increased ZNT1 expression and subsequent increased efflux of zinc ions cause an elevation of zinc ions in the extracellular space that might initiate the deposition of Aβ resulting in the creation of β-amyloid plaques.

All ZNTs (ZNT2-8), except for ZNT1, are localized on the intracellular membranes and are involved in transporting zinc ions into different intracellular compartments when the intercellular level of zinc ions is elevated [33]. ZNT3 is mainly localized in the membranes of zinc-rich synaptic vesicles of the ZEN terminals and serves to sequester zinc ions into the vesicles [75]. The vesicular zinc ions are released into the synaptic cleft during synaptic activity and interact with amino acid receptors on the synaptic plasma membrane [75]. Apart from the vesicular membranes of glutamatergic vesicles, ZNT3 is also expressed in the epithelial cells of the choroid plexus [74], the Bergman glial cells of mouse cerebellar cortex [73] and the postganglionic neurons of mouse superior cervical ganglia [72]. ZNT3 knockout mice are devoid of zinc ions in the ZEN terminals [14]. Synaptic release of zinc ions has been suggested to cause Aβ to precipitate into amyloid [9,26,31,39]. In addition, it has been shown that genetic ablation of ZNT3 in the Tg2576 Alzheimer mouse model inhibits the formation of senile plaques and greatly decreases zinc ion concentrations in the cerebral amyloid angiopathy (CAA) [26,39]. Taken together, our finding of an abundant expression of ZNT3 in the zinc-containing plaques and amyloid angiopathic vessels supports the notion that ZNT3 plays a key role in the formation of senile plaques and CAA.

Aβ accumulation has been reported in the endosomal/lysosomal system in postmortem AD brains [68], and the intraneuronal Aβ accumulations seem to precede amyloid plaque formation in APP transgenic mice [61,76]. ZNT4 might have an important role in this process as it is localized in the intracellular vesicular membrane and functions to increase vesicular zinc concentration [35,48]. In fact, in addition to bind to zinc, ZNT4 also binds to other divalent cations [50]. Intriguingly, many divalent cations, such as copper and iron, have been hypothesized to be involved in the pathogenesis of AD due to their abundant presence in the senile plaques [2,6,42,49]. This points to multiple roles of ZNT4 in the deposition of Aβ.

Aβ is generated from the amyloid precursor protein (APP) by a proteolytic activity of β- and γ-secretase [34]. APP and γ-secretase are also involved in the cerebral cholesterol shuttle, and the neurosecretases including γ-secretase provide strategies to treat sporadic and familial Alzheimer disorders [10,46]. The γ-secretase complex contains both high and low affinity zinc-binding sites. It catalyzes its substrates in the trans-Golgi network (TGN) [3]. Interestingly, the functions of ZNT5, ZNT6 and ZNT7 are believed to facilitate the translocation of the cytoplasmic zinc ions into the Golgi apparatus [12,32,36]. Therefore, we hypothesize that ZNT5-7 may be participating in the pathogenetic process of AD by transporting the cytoplasmic zinc ions into the Golgi apparatus and incorporating them into newly synthesized metalloproteins or influencing the activity of the metalloproteins, including the γ-secretase complex. Further studies are needed to identify the exact locations of these zinc transporters along the secretory pathway and roles of individual transporters in incorporation of zinc into metalloproteins.

Overall, the present study provides morphological evidence that zinc and ZNTs are involved in the pathological accumulation of Aβ. Although the intensity of ZNTs staining in the plaques differed somewhat among different transporters, they were found to be more abundant in the plaques than in the surrounding tissue, suggesting that ZNTs are involved in a complicated mechanism that leads to plaque formation. We hope that the present data add to the growing knowledge of the significance of ZNTs in the normal and AD brain.

Disclosure statement

We state that there are no potential conflicts of interest, including any financial, personal or other relationships with people or organizations that could inappropriately influence the current study.

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